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(54) **Genetic transformation using a PARP inhibitor**

(57) The invention concerns a process for producing transgenic eucaryote cells, particularly plants, which comprises: contacting a culture of untransformed cells with an inhibitor of poly-(ADP-ribose) polymerase for a period of time sufficient to reduce the response of the cultured cells to stress and to reduce their metabolism. The untransformed cells are then contacted with foreign DNA comprising at least one gene of interest under conditions in which the foreign DNA is taken up by the untransformed cells and the gene of interest is stably integrated in the nuclear genome of the untransformed cells to produce the transgenic cells. Optionally, the transgenic cells are recovered from the culture. Preferably, the inhibitor is niacinamide, preferably at a concentration of about 200 mg/l to 500 mg/l and the untransformed cells are cultured in a medium containing the inhibitor for a period of time of approximately 3 to 14 days prior to the contacting with the foreign DNA. The invention also relates to a plant having in the nuclear genome of its cells foreign DNA integrated only in the regions of the nuclear genome that are transcriptionally active in the cells of the plant when the cells are treated with an effective amount of a PARP inhibitor for a period of time sufficient to reduce cell metabolism to a state where gene expression is essentially limited to genes expressed irrespective of the differentiated or physiological condition of the cell.

EP 0 757 102 A1

Description

This invention is related to tissue culture of eucaryotic cells and improved techniques to obtain genetically transformed eucaryotic cells and organisms, such as transgenic plant cells or plants, by lowering the stress reaction of cultured eucaryotic cells prior to contacting the cells with foreign DNA, particularly by specific inhibition of poly-(ADP-ribose) polymerase.

Background to the invention

Over the years many techniques for the genetic transformation of higher organisms (animals and plants) have been developed. In these techniques it is the ultimate goal to obtain a transgenic organism, e.g. a plant, in which all cells contain a foreign DNA comprising a gene of interest (the so-called transgene) stably integrated in their genome, particularly their nuclear genome.

Transformation is a complex process which always involves the contacting of starting cells with a DNA, usually a DNA comprising foreign gene(s) of interest. The contacting of the cells with the DNA is carried out under conditions that promote the uptake of the DNA by the cells and the integration of the DNA, including the gene(s) of interest into the genome of the cell.

Starting cells for transformation are usually cells that have been cultured *in vitro* for some time. After contacting the cells with the DNA, the transformed cells generally need to be cultured *in vitro* for a certain period in order to separate the transformed cells from the non-transformed cells and, in the case of plants, to regenerate transformed plants from the transformed cells. Indeed, complete plants can be regenerated from individual transformed cells thus ensuring that all cells of the regenerated plant will contain the transgene.

In many plants, genetic transformation can be achieved by using the natural capacity of certain *Agrobacterium* strains to introduce a part of their Ti-plasmid, i.e. the T-DNA, into plant cells and to integrate this T-DNA into the nuclear genome of the cells. It was found that the part of the Ti-plasmid that is transferred and integrated is delineated by specific DNA sequences, the so-called left and right T-DNA border sequences and that the natural T-DNA sequences between these border sequences can be replaced by foreign DNA (European Patent Publication "EP" 116718; Deblaere et al, 1987 Meth.Enzymol. 153:277-293).

Certain plant species have proven to be recalcitrant to *Agrobacterium* mediated transformation and in these species, as well as in animals, genetic transformation has been achieved by means of direct gene transfer by which DNA is inserted into the cells by physical and/or chemical means, such as by electroporation, by treatment of the cells with polyethyleneglycol (PEG), by bombardment of the cells with DNA-coated microprojectiles, etc. (WO 92/09696; Potrykus et al, 1991, Annu.Rev.Plant Physiol.Plant Mol.Biol. 42:205-225).

Genetic transformation of eucaryotic cells is generally a random event, i.e. the transgene is integrated in the genome at random positions. Often several copies (or parts of copies) of the transforming DNA are integrated in a single position, and/or at different positions, resulting in a transformed cell containing multiple copies of the transgene. The expression of the transgene is known to be influenced by its position in the genome. For instance, a foreign DNA, when introduced in a plant cell appears to integrate randomly in the plant genome. Examination of independently transformed plants has shown a high degree of variability (up to 100-fold) in the expression level of the introduced gene. Several studies have shown no correlation between this "between-transformant variability" and the copy number of the introduced DNA at a given locus. It has been suggested that some of the variability in expression of introduced genes in transgenic plants is a consequence of "position effects" caused by influences of adjacent plant genomic DNA. Other factors that could contribute to the variability in expression are physiological variability of the plant material, differences in the number of independent T-DNA loci in different transformants or the inhibitory effects of certain T-DNA structures on gene expression. Between-transformant variability in expression has been observed for the majority of introduced genes in transgenic plants.

The variability in expression of many introduced genes in independent transgenic plants necessitates large numbers of transgenic plants to be assayed to accurately quantitate the expression of the gene. It would be of great importance if the amount of between-transformant variability could be reduced (Dean et al, 1988, NAR 16:9267-9283).

If the transgene is under the control of a tissue-specific promoter, with the expectation that it will be expressed in selected tissues of the transformed organisms, the position effects can lead, at least in some transformants, to loss of specificity of the promoter and expression of the transgene in undesired tissues, e.g. in tissue cultured *in vitro*.

Factors that are known to influence the efficiency and quality of the genetic transformation process are the method of DNA delivery, specific tissue culture conditions, the physiological and metabolic state of the target cells etc. Direct gene transfer methods for instance are generally known to result in transformed organisms with a high copy number of the transgene. Many of these factors are not under the control of man.

Summary of the Invention

This invention provides a process for producing transgenic eucaryotic cells, particularly plant cells. The process comprises contacting a culture of untransformed cells with an inhibitor of poly-(ADP-ribose) polymerase for a period of time sufficient to reduce the response of the cultured cells to stress and to reduce the metabolism of the cultured cells to stress and to reduce the metabolism of the cultured cells. The untransformed cells are then contacted with foreign DNA comprising at least one gene of interest under conditions in which the foreign DNA is taken up by the untransformed cells and the gene of interest is stably integrated in the nuclear genome of the untransformed cells to produce transgenic cells which are recovered from the culture.

The process may preferably comprise contacting untransformed eucaryotic (e.g. plant) cells with foreign DNA comprising at least one gene of interest under conditions in which the foreign DNA is taken up by the untransformed cells and the gene of interest is stably integrated in the nuclear genome of the untransformed cells to produce the transgenic cells. The untransformed cells are cultured in vitro in a culture medium containing an inhibitor of poly (ADP ribose) polymerase, preferably niacinamide, preferably for at least 2 to 3 days, particularly for at least 4 days (e.g. 4-5 days), before the contacting of the untransformed cells with said foreign DNA. The inhibitor can in addition also be applied to cultured cells that are being contacted or that have been contacted with the foreign DNA.

Description of the Invention

The present invention is based on the observations that poly-(ADP-ribose) polymerase (PARP) is an enzyme that is involved in regulating the general metabolic state of an eucaryotic cell and that inhibition of this enzyme can be used to influence the metabolic state of cells which are targeted for transformation (or which are being transformed) to increase the efficiency and/or quality of transformation.

In eucaryotes PARP is a monomeric nuclear Zn-finger protein of about 116 kD that is closely associated with nuclear DNA, particularly with actively transcribed euchromatic regions (Shah et al, 1995, Anal.Biochem. 227:1-13). The protein is normally an inactive enzyme but is known to be activated by nicked or otherwise damaged DNA. Active PARP transfers the ADP-ribose moiety of NAD⁺ to various nuclear proteins to synthesize a polymer of ADP-ribose bound to these proteins which include PARP itself, polymerases, histones, endonuclease etc. The proteins on which such a ADP-ribose polymer is synthesized become biologically inactive (de Murcia et al, 1994, TIBS 19:172-176; Cleaver et al, 1991, Mutation Res. 257:1-18).

The biological function of PARP is largely unknown but the enzyme has been implicated in:

- enhancement of DNA repair (Satoh et al, 1992, Nature 356:356-358; Satoh et al, 1993, J.Biol.Chem. 268:5480-5487),
- recombination events: in general inhibition of PARP is observed to inhibit illegitimate recombination and to increase intrachromosomal recombination but it does apparently not affect extrachromosomal recombination (Farzaneh et al, 1988, NAR 16:11319-11326; Waldman and Waldman, 1990, NAR 18:5981-5988; Waldman and Waldman, 1991, NAR 19:5943-5947),
- regulation of gene expression : inhibition of PARP is observed to decrease gene expression (Girod et al, 1991, Plant Cell, Tissue and Organ Culture 25:1-12);
- reducing the amount of available NAD⁺ (and by consequence its precursor ATP) : this results in a general slowing down of cell metabolism (Lazebnik et al, 1994, 371:346-347; Gaal et al, 1987, TIBS 12:129-130; Cleaver et al, supra)

It is known that PARP can be efficiently inhibited by a number of compounds (Durkacz et al, 1980, Nature 283:593-596; Sims et al, 1982, Biochemistry 21:1813-1821). Examples of such compounds are certain pyridine analogs such as nicotinamide analogs, including niacinamide, picolinamide, and 5-methyl nicotinamide; purine analogs like methylxanthines; thymidine; pyrazinamide analogs and many aromatic amides such as many benzamide analogs including benzamide, 3-methoxybenzamide and 3-aminobenzamide. For the purpose of this invention a PARP inhibitor is generally understood as any specific inhibitor of poly-(ADP-ribose) polymerase which can be taken up by a eucaryotic cell, particularly a plant cell, and which has a inhibition constant (K_i) which is lower than 1x10⁻⁵, particularly lower than 1x10⁻⁶. Generally it is desired that the PARP inhibitor used with this invention be a compound which in human lymphocytes, cultured in medium containing the inhibitor at a concentration of 2 mM, results in a 80-90 % inhibition of PARP (Sims et al, supra). Generally it is also preferred that cells cultured in medium containing the PARP inhibitor retain their capacity of DNA repair. Particularly preferred PARP inhibitors are those listed above and especially niacinamide (nicotinamide), picolinamide, 5-methylnicotinamide, 2-aminobenzamide, pyrazinamide, theobromine and theophylline. Particularly niacinamide is believed to be a useful inhibitor for the purpose of this invention.

Basically the present invention provides a modification of existing procedures for the genetic transformation of eucaryotic cells, particularly plant cells, by including in the medium in which such cells are cultured a PARP inhibitor

such as niacinamide, for a defined period of time. In particular the PARP inhibitor is added to the culture medium at least 1 day prior to the moment (the "contacting time") at which the cells are contacted with foreign DNA comprising one or more genes of interest. However, the PARP inhibitor may also be added to the culture medium during and after the contacting time.

5 In one aspect of this invention treatment of cultured cells, tissues or organs with PARP inhibitors can be used to increase the quality of transformation as measured by the copy number of the transgene and by variation in transgene expression (quality and quantity) in the transformed cells and in organisms obtained from the transformed cells. In many conventional procedures for genetic transformation of eucaryotic cells, particularly plant cells, cultured cells, tissues or organs are used as starting material and cells in such cultures are contacted with foreign DNA comprising at least one gene of interest (i.e. the transgene) under conditions that will promote the uptake of the foreign DNA in the cells and the ultimate integration of the foreign DNA into the genome of the cells. In one embodiment of the invention it is preferred that a PARP inhibitor is added to the culture medium for a period of at least 2-3 days, preferably at least about 3 days, prior to contacting the cells with the foreign DNA. The exact period in which the cultured cells are incubated in PARP inhibitor containing medium is believed not to be critical but should probably not exceed 4 weeks. It appears that 2-14 days, particularly 3-10 days, is an optimal period and best results were obtained with an incubation period of approximately 4 to 5 days prior to the contacting time. Generally it is believed that 4 days is a useful period for the PARP inhibitor to be added to the culture medium prior to the contacting time.

The concentration of the PARP inhibitor in the medium is also believed to have an effect on the inhibitor of PARP, which varies depending on the nature of the cells (species, tissue explant, general culture conditions, etc.) However, within certain concentration ranges, the effect is minimal, especially when the cultured cells are not incubated for longer than 14 days. The optimal concentration will depend on the nature of the specific PARP inhibitor used, particularly on its strength of inhibition (as measured by its K_i and/or by its percentage inhibition of PARP under standard conditions - Sims et al. *supra*). It was found for instance that the optimal concentration for nicotinamide is approximately 250 mg/l (i.e. about 2 mM) but it is believed that concentrations up to 1000 mg/l (approx. 8 mM) and as low as 150 mg/l (approx. 1.25 mM) can be used to good effect. Preferably the nicotinamide concentration should be between 200 and 300 mg/l, i.e. between approximately 1.5 mM and 2.5 mM. Similar concentrations apply to other PARP inhibitors. If incubation times of longer than 14 days are used it is believed that the PARP inhibitor concentration should be reduced below 2 mM (e.g. between 0.5 mM and 1.5 mM and particularly approximately 0.8 mM). For other PARP inhibitors optimal concentrations can be easily established by experimentation in accordance with this invention.

During transformation it is not known whether the integration of the DNA into the genome of the cell occurs immediately after uptake of DNA by the cell. It may very well be that the foreign DNA exists as free DNA within the cell for a certain period after the contacting time. Therefore cultured cells may be further incubated in medium containing a PARP inhibitor during and, for a limited period of time after, contacting the cells with the foreign DNA. Again the length of the incubation period is not critical but is preferably 2-10 days, particularly approximately 4 days. It is preferred that the inhibitor concentration of the PARP inhibitor in the culture medium after the contacting time should be below 2 mM, between 0.8 and 1 mM. If the cells that are to be transformed are not obtained from a cell or tissue culture (e.g. wheat intact tissue of an organism is contacted directly with DNA, as for example described in WO 92/09696) the PARP inhibitor may still be applied to the target cells prior to the contacting time but the addition of the PARP inhibitor to the culture of the transformed cells during or after the contacting time is preferred.

As indicated above, PARP inhibitor treatment of cultured cells for at least 2-3 days increases the quality of transformation. Indeed the number of copies of the foreign DNA is generally lower and variation in expression profile (level - i.e. the quantity - of expression as well as spatial and time distribution - i.e. the quality - of expression in the transgenic organism) of the gene(s) of interest in the foreign DNA, due to position effects, is decreased. However, at least in this aspect of the invention, the efficiency of transformation can be decreased. The efficiency of transformation as used herein can be measured by the number of transformed cells (or transgenic organisms grown from individual transformed cells) that are recovered under standard experimental conditions (i.e. standardized or normalized with respect to amount of cells contacted with foreign DNA, amount of delivered DNA, type and conditions of DNA delivery, general culture conditions etc.).

50 Therefore it is preferred that the invention is used with transformation procedures that already have a high efficiency, such as *Agrobacterium* mediated transformation of dicots and direct gene transfer in monocots, particularly cereals (e.g. electroporation or particle bombardment of compact embryogenic callus in cereals - see WO 92/09696). Indeed these transformation procedures are generally highly efficient but the quality of transformation is generally poor. Position effects are large and, especially with direct gene transfer, the copy number of the transgene is often exceptionally high making analysis and selection of optimal transformants, as well as further breeding with the transformants, difficult.

55 In another aspect of this invention treatment of cultured plant cells for a short period of time (i.e. 1 day to maximally 2 days) prior to contacting the cells with DNA may be used to increase the efficiency of *Agrobacterium* mediated transformation of plant species, such as many monocots, particularly the major cereals such as rice, wheat and corn, for which this method is generally inefficient. In this embodiment of the invention the contacting of the plant cells with the

DNA should of course be understood as contacting the cells with an appropriate Agrobacterium strain harboring an artificial T-DNA containing the foreign DNA with the gene(s) of interest. In this embodiment of the invention the quality of transformation is expected not to be affected but this is generally deemed to be of lesser importance since Agrobacterium mediated transformation, being a biological process, already results in a generally low copy number of the transgene in the transformed plant cells.

In accordance with this invention the addition of PARP inhibitors, such as niacinamide, to the culture medium of eucaryotic cells, can be used in combination with any known transformation procedure that requires cells, tissues or organs cultured in vitro as starting cells to be contacted with foreign DNA. The process of this invention is thus generally identical to existing conventional transformation methods except for the fact that at some times during the tissue culture of the cells, a PARP inhibitor is added to the culture medium.

The cell of a plant, particularly a plant capable of being infected with Agrobacterium such as most dicotyledonous plants (e.g. Brassica napus) and some monocotyledonous plants, can be transformed using a vector that is a disarmed Ti-plasmid containing the gene(s) of interest and carried by Agrobacterium. This transformation can be carried out using conventional procedures (EP 0,116,718; Deblaere et al, *supra*; Chang et al, 1994, The Plant Journal 5:551-558). Preferred Ti-plasmid vectors contain the foreign DNA between the border sequences, or at least located to the left of the right border sequence, of the T-DNA of the Ti-plasmid. Of course, other types of vectors can be used to transform the plant cell, using procedures such as direct gene transfer (as described, for example, in EP 0,233,247), pollen mediated transformation (as described, for example, in EP 0,270,356, PCT patent publication "WO" 85/01856, and US patent 4,684,611), plant RNA virus-mediated transformation (as described, for example, in EP 0,067,553 and US patent 4,407,956) and liposome-mediated transformation (as described, for example, in US patent 4,536,475). Cells of monocotyledonous plants such as the major cereals including corn, rice, wheat, barley, and rye, can be transformed (e.g. by electroporation) using wounded or enzyme-degraded intact tissues capable of forming compact embryogenic callus (such as immature embryos in corn), or the embryogenic callus (such as type I callus in corn) obtained thereof, as described in WO 92/09696. In case the plant to be transformed is corn, other recently developed methods can also be used such as, for example, the method described for certain lines of corn by Fromm et al, 1990, *Biol/Technology* 8:833; Gordon-Kamm et al., 1990, *Bio/Technology* 2:603 and Gould et al, 1991, *Plant Physiol.* 95:426. In case the plant to be transformed is rice, recently developed methods can also be used such as, for example, the method described for certain lines of rice by Shimamoto et al., 1989, *Nature* 338:274; Datta et al., 1990, *Biol/Technology* 8:736; and Hayashimoto et al., 1990, *Plant Physiol.* 93:857; Hiei et al, 1994, *The Plant Journal* 6:271-282).

The transformed cell can be regenerated into a mature plant and the resulting transformed plant can be used in a conventional breeding scheme to produce more transformed plants with the same characteristics or to introduce the gene(s) of interest in other varieties of the same related plant species. Seeds obtained from the transformed plants contain the chimeric gene(s) of this invention as a stable genomic insert. Thus the gene(s) of interest when introduced into a particular line of a plant species can always be introduced into any other line by backcrossing.

In animals pluripotent embryonic or somatic stem cells can be used as target for transformation (Capecchi et al, 1989, *TIG*:5:70-76).

The transformed cells and organisms of any plant or animal species, produced by the process of this invention, contain the foreign DNA as a stable insert in their genome, particularly in regions of the genome that remain transcriptionally active in the untransformed cells that have been exposed to a PARP inhibitor in accordance with this invention. As described above it is believed that in cells treated with a PARP inhibitor for at least 3 days, particularly for at least 4 days, only a limited number of genomic regions remain transcriptionally active. In this regard the transformed cells, obtained with this process of the invention, are characterized by having the foreign DNA integrated in a limited number of genomic regions. That the transformed cell or organism was obtained by this process of the invention can thus be easily ascertained by 1) culturing transformed cells or tissues under conditions that are similar to those in which the untransformed cells or tissues were grown or incubated prior to the integration of the foreign DNA in the genome (i.e. incubating in medium containing 250 mg/l niacinamide for 4-5 days prior to the contacting time), and 2) monitoring the expression of at least one transgene in the foreign DNA that is expected to be expressed under normal tissue culture conditions (i.e. a selectable marker gene under the control of a promoter that directs expression in tissue culture). Under the above conditions the transformed cells or tissues of this invention express the relevant transgene in the tissue culture at essentially the same levels whether or not a PARP inhibitor is present in the culture medium. It is thus expected that, for instance after 4-5 days of culturing of the transformed cells in medium containing the PARP inhibitor, mRNA levels are not significantly decreased, i.e. do not become lower than 75%, preferably not become lower than 90%, when compared to the mRNA levels observed in cells cultured in medium not containing the inhibitor. Indeed, if the relevant transgene is integrated in other regions of the genome (i.e. in regions that are normally not transcriptionally active in cells treated with PARP inhibitor according to this embodiment of the invention), the expression of the relevant transgene is considerably reduced after incubation of the cells in medium containing the PARP inhibitor for at least 3 days, e.g. 4-5 days (i.e. mRNA levels will drop below 75%, particularly below 50%, more particularly below 30% when compared to the mRNA levels observed in cells cultured in medium not containing the inhibitor).

The method of the present invention can in principle be used to transform eucaryotic cells with any foreign DNA.

Generally the foreign DNA comprises at least one gene of interest comprising 1) a promoter region with a promoter capable of directing transcription of DNA into a RNA in cells of the eucaryotic, e.g. plant, species that is to be transformed and 2) a coding region coding for a RNA or protein. Most often the gene of interest will also comprise 3) a 3' untranslated region of a eucaryotic gene containing a polyadenylation signal.

5 The promoter can be selected to direct expression in selected tissues of the eucaryotic organism. Such a tissue-selective promoter is not expected to direct expression in other non-selected tissues. For instance promoters are known that direct expression selectively in stamen tissues of a plant and such promoters have been used to produce male sterile plants and other plants useful for producing hybrids (EP 344029; EP 412911; WO 9213956; WO 9213957; Mariani et al, 1990, Nature 347:737-741; Mariani et al, 1992, Nature 357:384-387).

10 It is believed that the method of the present invention is particularly useful to transform eucaryotic cells with at least one gene of interest comprising a tissue-selective promoter, such as a stamen selective promoter, especially if expression of the gene of interest in the organism, such as a plant, outside the selected tissues (where the tissue-selective promoter is active, i.e. directs expression) is undesired for example because the gene product (for instance a protein such as a ribonuclease, e.g. barnase) is capable of killing or disabling the cells in which they are produced. In such cases
15 expression of the gene of interest in tissue culture, or in non-selected tissues of the organisms can negatively affect the quality as well as the apparent efficiency of transformation. When the method of this invention is used, the overall efficiency of transformation may be reduced but the average quality of transformation is expected to be significantly improved because of lower copy number of the gene of interest in the genome of the transformed cells and because of reduced position effects i.e. the general integration of the gene of interest in the genomes at locations that minimally affect the transcriptional properties of the promoter of the transgene.

20 The foreign DNA used in the method of this invention will generally also comprises a selectable marker gene the expression of which allows the selection of transformed cells (or organisms) from non-transformed cells (or organisms). Such selectable marker gene generally encodes a protein that confers to the cell resistance to an antibiotic or other chemical compound that is normally toxic for the cells. In plants the selectable marker gene may thus also encode a
25 protein that confers resistance to a herbicide, such as a herbicide comprising a glutamine synthetase inhibitor (e.g. phosphinothricin) as an active ingredient. An example of such genes are genes encoding phosphinothricin acetyl transferase such as the *sfr* or *sfrv* genes (EP 242236; EP 242246; De Block et al, 1987 EMBO J 6:2513-2518).

The inventors also found that the initial reaction of cells particularly plant cells contacted with PARP inhibitors is a stress reaction which enhances free radical production by the cell. However, this stress only lasts for a limited period of
30 time after which further contact with the PARP inhibitor causes a decrease in cell metabolism. Therefore, the invention also relates to a new method to assess the agronomical fitness of a population of transformed plants to determine in which lines the plants have a foreign DNA integrated in their genomes in a way that agronomical performance is not or substantially not affected. The assay is based on the comparative reaction of transgenic cells and corresponding untransformed controls to stress conditions.

35 The method comprises exposing the transgenic cells to stress conditions which induce the production of free radicals in the tissues of the cells, measuring the amount of free radicals produced in these tissues and comparing the amount of free radicals produced in the transgenic cells with the amount of free radicals produced in control cells exposed to similar stress conditions. Preferably the cells of the transgenic organism to be assayed are exposed to stress conditions by being treated with a substance which induces increasing osmotic and/or salt stress on the cells.

40 The properties of PARP inhibitors, such as niacinamide, to enhance free radical production in cells incubated with the inhibitor for not longer than 2 days, preferably not longer than 1 day, can be used to assay the (relative) fitness of a population of transgenic eucaryotic organisms, particularly plants.

The term fitness used herein is intended to designate the agronomical performance of a population of plants, as measured for instance by its yield (e.g. its seed yield) as compared to a given reference population. Agronomical performance is generally thought to be correlated with the general resistance of the plants to a range of stress conditions
45 which are likely to be encountered in the field locations where the plants are normally grown. For any population of transformed plants (i.e. a transgenic line) the relevant reference population is a population of untransformed plants of the same variety.

It is known that in transformed plants and other organisms transgene expression may be qualitatively and quantitatively influenced by the genomic domain in which the transgene(s) are integrated, that undesired transgene expression may interfere with cell metabolism (e.g. when the transgene encodes a cytotoxic protein), that mutations may be induced in the transformed organism either by somaclonal variation or by insertional inactivation of endogenous genes by the transgene(s), or that expression of endogenous genes may be deregulated by sequences in the foreign DNA. As a consequence many transformed lines may not be agronomically useful.

55 The assay of this invention will for example allow to identify a line (i.e. a group of genetically similar plants) of transformed plants that have the transgene(s) integrated in regions that minimally affect the fitness of the plants, thus avoiding the extensive laboratory, greenhouse and/or field evaluations which are normally required to identify the transformants with the best agronomical properties.

The assay in accordance with this invention essentially comprises the incubation of cells or tissues of transformed

plants of a particular transgenic line (e.g. callus, hypocotyl explants, shoots, leaf discs, whole leaves etc.), preferably with a PARP inhibitor (although for some plant species this is not necessary), under a range of conditions which induce the production of free radicals in the tissues. An incubation time of approximately one day is normally sufficient to generate the desired amount of free radicals. Appropriate controls, i.e. untransformed tissues obtained from untransformed plants at the same developmental stage and grown in the same conditions as the transformed plant from which the transformed tissue was obtained, are subjected to the same treatment. Preferably the untransformed line is identical to the transgenic line except for the presence of the transgene(s). For each plant line (control or transformant) it is preferred that a number of plants is assayed.

Useful conditions for the incubation of the untransformed and transformed tissues are those which induce increasing osmotic and salt stress in the incubated cells or tissues. For example a series of buffers with different salt concentrations containing a PARP inhibitor can be made. A useful buffer series is a K-phosphate buffer containing 2% sucrose and 250 mg/l niacinamide in which the K-phosphate concentration is increased from anywhere between 10 to 80 mM (e.g. in steps of 5 mM, i.e. 10, 20, 25, 30, 35, 40, 45, 50, 55, 60 mM). The K-phosphate concentration induces mild but increasing salt and osmotic stress in plant cells. The niacinamide in the medium enhances radical production and stress on the plant cells. The range of K-phosphate concentrations used depends on the natural sensitivity of the plant species (or if desired the plant line) to the salt and osmotic stress. In sensitive plant species, which do not tolerate high salt stress, the maximum K-phosphate concentration may for instance be 50 mM, in less sensitive species this maximum K-phosphate concentration can be increased up to 70 or 80 mM or even higher. For each plant species the minimum and particularly the maximum salt (e.g. K-phosphate) concentration can be determined experimentally for an untransformed line - the only requirement is that at all concentrations used the plant tissue remains viable. Although the addition of a PARP inhibitor to the medium, such as niacinamide, is preferred it is not required for assaying plant species that are very sensitive to salt and/or osmotic stress.

After the one day incubation the capacity of the transformed and control tissues to reduce 2,3,5-triphenyltetrazolium chloride (TTC) is measured e.g. by the following procedure which is modified from Towill and Mazur (*supra*):

- incubate the tissues for 1 to 4 hours in K-phosphate buffer (pH 7.4) containing 10 mM TTC and 0.1 % Tween20. As a control similar plant material is incubated in the same buffer without TTC.
- extraction of reduced TTC (e.g. freezing at -70°C followed by thawing at 40°C and shaking the plant material in ethanol for 45-60 minutes)
- spectrophotometric quantification of reduced TTC at 485 nm. The O.D. of the control extract is subtracted from the O.D. of the TTC-reacted extracts. In the above conditions 0.1 mM reduced TTC corresponds to an O.D. of 0.214 (light path 1 cm)
- the reducing capacity of the transformed plant line is compared to that of the control line.

The amount of reduced TTC is determined by the intensity of the cytochromal and alternative respiratory pathways and the radical concentration in the tissues which, in turn are determined by the presence of mutations, the expression of genes affecting the metabolic activity of the plant cells, the developmental stage and the reaction of the tissue to external factors, such as stress factors.

The TTC reducing capacity (as for instance measured by the O.D. at 485 nm) for tissues incubated at high salt concentration (TTC-high) is expressed as the percentage of the TTC reducing capacity of the tissues incubated at a low salt concentration (TTC-low); in other words a TTC-ratio value is calculated as follows:

$$\text{TTC-ratio} = \text{TTC/high} \cdot 100 / \text{TTC/low}.$$

The value of TTC-ratio is a measure of the fitness of a plant line as compared to a control line.

The determination of TTC-low and TTC-high will depend on the sensitivity of the plant species to the applied salt stress. Usually TTC-low will correspond to a salt concentration between 10 and 25 mM K-phosphate, e.g. at 20 mM while TTC-high will correspond to a salt concentration between 50 and 80 mM K-phosphate. The only requirement is that TTC-high should be significantly lower than TTC-low; preferably TTC-high should be lower than 50% of TTC-low, particularly lower than 30% of TTC-low. For instance for *Brassica napus*, TTC-low and TTC-high can be typically obtained from tissues incubated at respectively 20mM and 60 mM K-phosphate buffer containing 250 mg/l niacinamide. TTC-high and TTC-low, for both the transformed and untransformed line, will usually be an average obtained from several measurements taken on a number of tissue explants from a number of plants of each line. For instance for each line of *Brassica napus* about 32 leaf discs (diameter 1 cm) from 8 different plants (i.e. about four leaf discs per plant) can be assayed to determine 32 TTC-high and 32 TTC-low values which are averaged to obtain the TTC-high and TTC-low values used for the calculation of TTC-ratio. Transformed lines with a value of TTC-ratio which does not deviate more than 20%, preferably not more than 10% of the TTC-ratio value of the control line are selected. These lines are likely to have the transgene(s) integrated in regions that minimally affect the fitness of the plants.

Unless otherwise indicated all experimental procedures for manipulating recombinant DNA were carried out by the

standardized procedures described in Sambrook et al., 1989, "Molecular Cloning: a Laboratory Manual", Cold Spring Harbor Laboratory, and Ausubel et al., 1994, "Current Protocols in Molecular Biology", John Wiley & Sons. The polymerase chain reactions ("PCR") were used to clone and/or amplify DNA fragments. PCR with overlap extension was used in order to construct chimeric genes (Horton et al., 1989, Gene 77:61-68; Ho et al., 1989, Gene 77:51-59).

- 5 All PCR reactions were performed under conventional conditions using the Vent™ polymerase (Cat. No. 254L - Biolabs New England, Beverly, MA 01915, U.S.A.) isolated from *Thermococcus litoralis* (Neuner et al., 1990, Arch.Microbiol. 153:205-207). Oligonucleotides were designed according to known rules as outlined for example by Kramer and Fritz (1968, Methods in Enzymology 154:350), and synthesized by the phosphoramidite method (Beaucage and Caruthers, 1981, Tetrahedron Letters 22:1859) on an applied Biosystems 380A DNA synthesizer (Applied Biosystems B.V., Maarsen, Netherlands). In the examples MS medium means Murashige and Skoog medium (Murashige and Skoog, 1962, Physiol. Plant 15:473-479).

In the following examples, reference will be made to the following sequence listing and figures:

Sequence Listing

- 15 SEQ ID NO 1 : T-DNA of plasmid pTHW107
 SEQ ID NO 2 : plasmid pTS172
 SEQ ID NO 3 : PT72 promoter contained in plasmid pTS772

20 Examples

Example 1 : Tissue culture of wheat embryogenic callus and Brassica napus hypocotyl explants in media containing a PARP inhibitor.

- 25 Wheat embryogenic callus was cultured on W2 medium (see Example 2). When niacinamide (a potent PARP inhibitor) was added to the medium at a concentration of 250 mg/l (approx. 2 mM) it was observed that after 4 days the growth of the tissue was slowed down considerably (to approximately 30% of the normal rate after 4 weeks) but the tissue remained viable for extended periods of time (i.e. at least one month). If niacinamide was subsequently removed from the medium the tissue started to grow normally again. It was also observed that after 4-5 days of incubation of the plant tissue with niacinamide, the TTC-reducing capacity (Towill and Mazur, 1975, Can J.Bot. 53:1097-1102) of the tissue was substantially decreased probably indicating a reduction of the production of free radicals and decreased mitochondrial electron transport.

- Similar observations were made when *Brassica napus* hypocotyl explants were cultured on A5 medium (see Example 3) containing 250 mg/l niacinamide. It was also observed that, in *Brassica napus* tissue cultured on medium containing niacinamide, no anthocyanin was produced; normally anthocyanin in tissue culture is produced in stress conditions. In addition it was observed that after 4-5 days of incubation of the plant tissue with niacinamide, the concentrations of hydroxyl free radical and dehydroascorbate in the explants were drastically decreased. It was also observed that, after a 4 day incubation in niacinamide containing medium, the percentage of cultured cells that were in G2 phase of the cell cycle was considerably increased (up to 45 % of all cells in the culture).

- 40 The above observations are interpreted as indicating that treating cultured cells with a PARP inhibitor for about 4-5 days generally results in:

- 1) a significant reduction of the response of the cultured cells to stress as measured for instance by free radical and/or anthocyanin production, and
- 45 2) a reduction of the general metabolism of the cultured cells to a very basic level as indicated by the fact that the tissue growth was slowed down, and the TTC reducing capacity was decreased while the tissue remained viable.

- It is inferred that under these conditions many genes in cells (e.g. cultured cells) that would normally be switched on in response to stress (such as during transformation conditions) are in fact no longer induced. It is expected that in such cells which only display a very basic metabolism, general "housekeeping genes", i.e. genes that are expressed in any cell irrespective of its differentiated state or metabolic or physiological condition, are expressed.

- As it is believed that foreign DNA is preferably inserted in portions of the genome that are transcriptionally active it follows that treatment with PARP inhibitors will condition eucaryotic cells to incorporate any foreign DNA preferentially in genomic regions which are transcribed in all cells and not in regions of the genome which would only be transcribed under certain conditions, i.e. stress conditions. This means that the number of locations in which foreign DNA is integrated, and the concomitant variation in expression profile of the transgene(s), is reduced.

It is further believed that this enhances integration of foreign genes of interest in such locations which in turn results in a more reliable and faithful expression of these genes which are less affected by cell differentiation or cell physiological and biochemical changes due to for instance environmental conditions.

Example 2 : Transformation of wheat with a barnase gene under the control of a stamen-specific promoter using the particle bombardment

The Wheat Spring variety Pavon was grown in a greenhouse or conditioned room at 23-24°C during daytime and 18-20°C at night, with a photoperiod of 16 hours light and 8 hours dark. Developing seeds (white-greenish with white semi-liquid endosperm) were harvested, sterilized by incubation for 1 minute in 70% ethanol followed by 15 minute incubation in 1.3% NaOCl+ 0.1% Tween 20, and washed with sterile water. The sterilized seeds were either used directly or were stored for one day at 4-7°C.

Immature embryos of about 1 mm in size were isolated and were placed, with the scutellum upwards, on callus inducing medium W1 (MS medium supplemented with 3% sucrose, 40 mg/l adenine.SO₄, 0.5 mg/l thiamine.HCl, 0.5 g/l 2-[N-Morpholino] ethane sulfonic acid (Mes) pH 5.8, 0.5% agarose, 0.5 to 2.5 mg/l CuSO₄.5H₂O, 25 mg/l acetylsalicylic acid and 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D)) and were incubated for 3 weeks at 27°C in the dark.

Embryogenic sections of the developing callus were isolated, placed on callus maintenance medium W2 (W1 medium but without acetylsalicylic acid and with only 0.5 mg/l CuSO₄.5H₂O and 1 mg/l 2,4-D), and incubated for 3 weeks at 24-25°C in the light (approx. 20 mEinsteins/s/m² (with a photoperiod of 16 hours light and 8 hours dark).

About 2 weeks prior to bombardment the calli were cleaned up by removal of non-morphogenic (i.e. the nonembryogenic and nonmeristematic) parts and were subcultured on W2 medium.

For bombardment the calli were divided into small pieces with an average maximum diameter of about 3 mm. These pieces were placed at the center of a 9 cm Petridish containing W2 medium in a circle with a diameter of approx. 0.5 cm. In tissues that were treated with niacinamide, niacinamide (250 mg/l) was added to the W2 medium and the tissue pieces were maintained under these conditions for 4 days after they were bombarded. Bombardment was carried out using the Biolistic PDS-1000/He apparatus (Bio-Rad). Preparation of the microcarriers (0.4-1.2µm) and the coating of the microcarriers with DNA was essentially carried out according to the manufacturer's instructions. The Petridishes containing the calli were placed at level 2 of the apparatus and the bombardment was done at 1550 psi.

For the transformation experiments the following plasmid DNA was used.

- plasmid pTS172 the sequence of which is given in SEQ ID No 2. This plasmid contains the following chimeric genes:

- P35S-bar-3'g7
- PE1-barnase-3'nos

in which in which P35S is the 35S promoter of the Cauliflower Mosaic virus, bar is a DNA encoding phosphinothricin acetyltransferase (EP 242236), 3'g7 is the 3' untranslated end of the Agrobacterium T-DNA gene 7, PE1 is a stamen-specific promoter from rice gene E1 (WO 9213956), barnase is a DNA encoding barnase (Hartley, 1988, J.Mol.Biol.202:913-915), and 3'nos is the 3' untranslated end of the Agrobacterium T-DNA nopaline synthase gene,

- plasmid pTS772 which is identical to pTS172 except that the region between nucleotides 2625-4313 of pTS172, containing PE1, is replaced by the sequence of SEQ ID No 3 containing the PT72 promoter. Thus, plasmid pTS772 contains the following chimeric genes:

- P35S-bar-3'g7
- PT72-barnase-3'nos in which PT72 is a stamen-specific promoter from rice gene T72 (WO 9213956)

The bombarded calli were transferred to selective medium W2 containing 2.5 mg/l phosphinothricin (PPT) and, if necessary, 100 mg/l niacinamide. The calli that were placed on medium containing niacinamide were transferred after 4 days to niacinamide-free W2 medium containing 2.5 mg/l PPT. The cells were cultured at 24-25°C.

After two weeks the calli were subcultivated on W2 medium and after a further two weeks the growing parts of the calli were transferred to regeneration medium W4 (W1 medium but without acetylsalicylic acid and with only 0.5 mg/l CuSO₄.5H₂O and 0.5 mg/l 2,4-D). Calli were subcultivated every two weeks at which time the nonmorphogenic parts of the calli were removed. When the call started to form shoots they were transferred to W5 medium (W1 medium with half concentrated MS medium and only 0.5 mg/l CuSO₄.5H₂O and without acetylsalicylic acid and 2,4-D, but supplemented with 50 mg/l myo-inositol, 0.25 mg/l pyridoxine.HCl and 0.25 mg/l nicotinic acid) containing 2.5 mg/l PPT. For the rest of the procedure temperature was maintained at a maximum of 23°C. The calli were subcultivated every 3-4 weeks. Once the shoots started to elongate and small roots started to form, the whole calli (or if possible individual shoots) were transferred to 1 liter vessels with W6 medium (half-concentrated MS medium supplemented with 1.5% sucrose, 50 mg/l myoinositol, 0.25 mg/l pyridoxine.HCl, 0.25 mg/l nicotinic acid, 0.5 mg/l thiamine.HCl, 0.7% agar (Difco) pH 5.8 and 0.5 mg/l CuSO₄.5H₂O) containing 2.5 mg/l PPT. Once the shoots and roots had grown out, individual shoots were separated from each other and transferred to 1 l vessels containing W6 medium with 2.5 mg/l PPT. Well developed shoots

are tested for PPT resistance by means of the TLC assay (De Block et al, 1987, EMBO 6:2513-2518) or by direct assay of ammonium production in the tissue. Transformed shoots were finally transferred to the greenhouse into soil.

For analysis of the results the transformed plants could be subdivided according to the niacinamide treatment of the parent calli during tissue culture. Thus the following groups were distinguished:

Group	Niacinamide treatment
None	No treatment
Before 250	250 mg/l niacinamide for four days prior to bombardment
Before/After	250 mg/l niacinamide for four days prior to bombardment plus 100 mg/l niacinamide for four days after bombardment

Results of the experiments are presented in Tables 1 and 2. Plants could be obtained only from bombarded calli that were treated with niacinamide. For the plants that were transformed with plasmid pTS172 it was demonstrated that the foreign DNA, comprising the chimeric PE1-barnase-3'nos and P35S-bar-3'g7, was stably incorporated in the wheat genome in 2 to 3 copies on the average. The plants transformed with plasmid pTS772 are still being analyzed.

The fact that variation in expression profile (e.g. tissue-specificity) of the transgenes, especially the chimeric barnase genes, was decreased in transformed cells was evident from the fact that male-sterile plants that otherwise looked completely healthy could be obtained only from bombarded calli treated with niacinamide. It is believed that this is due to a more faithful expression characteristics (i.e. lack of expression) of the integrated stamen-selective barnase gene in these calli and shoots regenerated from these calli. In the control calli undesired expression of the barnase gene in tissue cultured cells might have prevented recovery of any transformed plants from these calli. It is expected that to obtain the same number of male-sterile wheat plants from control calli a much larger number of calli would have to be bombarded.

Results of wheat transformation experiments

Table 1

Plasmid pTS172				
Treatment	Nr of bombarded calli	Nr of PPT-resist- antcalli recovered	Nr of PPT resistant plants recovered	Nr of MS plants recovered
None	60	30	1 ^{a)}	0
Before 250	125	30	3	3 ^{b)}

a) This plant proved to be fertile and transformed only with the chimeric bar gene.

b) The obtained plants looked healthy and tillered vigorously.

Table 2

Plasmid pTS772				
Treatment	Nr of bombarded calli	Nr of PPT-resistant calli recovered	Nr of PPT resistant plants recovered	Nr of MS plants recovered
None	250	22	0	0
Before 250	210	75	7	2 ^{a)b)}
Before/After	210	45	4	1 ^{b)}

a) The obtained plants looked healthy and tillered vigorously

b) Regenerated plants are still in the progress of being analyzed for male-sterility phenotype. In the Before 250 category only 2 plants could be scored for male-sterility phenotype.

Example 3: Transformation of oilseed rape with a barnase gene under the control of a stamen-specific promoter using *Agrobacterium* mediated transformation.

Hypocotyl explants of *Brassica napus* are obtained, cultured and transformed essentially as described by De Block et al, 1989, Plant Physiol. 914:694-701 except for the following modifications:

- hypocotyl explants are precultured for 3 days in A2 medium (MS, 0.5 g/l Mes (pH 5.7), 1.2% glucose, 0.5% agarose, 1 mg/l 2,4-D, 0.25 mg/l naphthalene acetic acid (NAA), 1 mg/l 6-benzylaminopurine (BAP)).
- infection medium A3 is MS, 0.5 g/l Mes (pH 5.7), 1.2% glucose, 0.1 mg/l NAA, 0.75 mg/l BAP, 0.01 mg/l gibberellin acid (GA3)
- selection medium A5 is 0.5 g/l Mes (pH 5.7), 1.2 % glucose, 40 mg/l adenine.SO₄, 0.5 g/l polyvinylpyrrolidone (PVP), 0.5% agarose, 0.1 mg/l NAA, 0.75 mg/l BAP, 0.01 mg/l GA3, 250 mg/l carbenicillin, 250 mg/l triacillin, 5 mg/l AgNO₃.
- regeneration medium A6 is MS, 0.5 g/l Mes (pH 5.7), 2% sucrose, 40 mg/l adenine.SO₄, 0.5 g/l PVP, 0.5% agarose, 0.0025 mg/l BAP, 250 mg/l triacillin.
- healthy shoots are transferred to rooting medium which was A8 : 100-130 ml half concentrated MS, 1% sucrose (pH 5.0), 1 mg/l isobutyric acid (IBA), 100 mg/l triacillin added to 300 ml perlite (final pH 6.2) in 1 liter vessels.

Hypocotyl explants (with or without niacinamide treatment) were infected with *Agrobacterium tumefaciens* strain C58C1Rif carrying T-DNA vector pTHW107 and a helper Ti-plasmid pMP90 (Koncz and Schell, 1986, Mol.Gen.Genet. 204:383-396)(or a derivative thereof).

Plasmid pTHW107 is a vector carrying a T-DNA comprising the following chimeric genes:

- PTA29-barnase-3'g7
- PSSU-bar-3'nos

in which PTA29 is the promoter of the TA29 gene of tobacco (EP 344029) and PSSU is the promoter of the gene of *Arabidopsis thaliana* encoding the small subunit of Rubisco. The complete sequence of the T-DNA of pTHW107 is presented in SEQ ID No 1.

For the niacinamide treated explants, niacinamide (250 mg/l) was added to the media for the last 4 days prior to infection with *Agrobacterium*. Plants regenerated from transformed calli obtained on niacinamide cultured cells are observed to have a low copy number as well as to display less variation in the expression profile of the transgenes.

All publications cited in this application are hereby incorporated by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: PLANT GENETIC SYSTEMS N.V.
 (B) STREET: Plateaustraat 22
 (C) CITY: Ghent
 (E) COUNTRY: Belgium
 (F) POSTAL CODE (ZIP): 9000
 (G) TELEPHONE: 32 9 235 84 58
 (H) TELEFAX: 32 9 224 06 94
 (I) TELEX: 11.361 Pgsgen

(ii) TITLE OF INVENTION: GENETIC TRANSFORMATION USING A PARP INHIBITOR

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: EP95401844.6

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4946 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: T-DNA of plasmid pTHW107

(ix) FEATURE:

(A) NAME/KEY: -
 (B) LOCATION: complement (1..25)
 (D) OTHER INFORMATION: /label= RB
 /note= "T-DNA right border"

(ix) FEATURE:

(A) NAME/KEY: -
 (B) LOCATION: complement (97..330)
 (D) OTHER INFORMATION: /label= 3'g7
 /note= "3' untranslated region containing the
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(A) NAME/KEY: -
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 /note= "region coding for phosphinothricin acetyl
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(ix) FEATURE:

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 (B) LOCATION:complement (883..2608)
 (D) OTHER INFORMATION:/label= PSSU
 /note= "promoter region of Rubisco small subunit gene of
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(ix) FEATURE:

(A) NAME/KEY: -
 (B) LOCATION:complement (2658..3031)
 (D) OTHER INFORMATION:/label= 3'nos
 /note= "3' untranslated region containing the
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(ix) FEATURE:

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 (B) LOCATION:complement (3032..3367)
 (D) OTHER INFORMATION:/label= barnase
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(A) NAME/KEY: -
 (B) LOCATION:complement (3368..4876)
 (D) OTHER INFORMATION:/label= PTA29
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(ix) FEATURE:

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 (D) OTHER INFORMATION:/label= LB
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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EP 0 757 102 A1

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55

EP 0 757 102 A1

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	AAAGTATATA AATATATATT TGGAAGCGAC TAAAAATAA CTTTCTCAT ATTATACGAA	4560
50	CCTAAAAACA GCATATGGTA GTTCTAGGG AATCTAAATC ACTAAAATTA ATAAAAGAAG	4620
	CAACAAGTAT CAATACATAT GATTTACACC GTCAAACACG AAATTCGTAA ATATTTAATA	4680

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TAATAAAGAA TTAATCCAAA TAGCCTCCCA CCCTATAACT TAACTAAAA ATAACCAGCG 4740
 AATGTATATT ATATGCATAA TTTATATATT AAATGTGTAT AATCATGTAT AATCAATGTA 4800
 TAATCTATGT ATATGGTTAG AAAAAGTAAA CAATTAATAT AGCCGGCTAT TTGTGTAAAA 4860
 ATCCCTAATA TAATCGCGAC GGATCCCCGG GAATTCGGG GAAGCTTAGA TCCATGGAGC 4920
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 6548 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: plasmid pTS172

(ix) FEATURE:
 (A) NAME/KEY: -
 (B) LOCATION:complement (2019..2288)
 (D) OTHER INFORMATION:/label= 3'nos
 /note= "3' untranslated region containing the
 polyadenylation signal of the nopaline synthase gene of Agrobacterium
 T-DNA"

(ix) FEATURE:
 (A) NAME/KEY: -
 (B) LOCATION:complement (2289..2624)
 (D) OTHER INFORMATION:/label= barnase
 /note= "region coding for barnase"

(ix) FEATURE:
 (A) NAME/KEY: -
 (B) LOCATION:complement (2625..4313)
 (D) OTHER INFORMATION:/label= PE1
 /note= "promoter region of E1 gene of rice"

(ix) FEATURE:
 (A) NAME/KEY: -
 (B) LOCATION:4336..5710
 (D) OTHER INFORMATION:/label= P35S
 /note= "35S promoter region of Cauliflower mosaic virus"

(ix) FEATURE:
 (A) NAME/KEY: -
 (B) LOCATION:5711..6262
 (D) OTHER INFORMATION:/label= bar
 /note= "region coding for phosphinothricin acetyl
 transferase"

EP 0 757 102 A1

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION:6263..6496

(D) OTHER INFORMATION:/label= 3'g7

/note= "3' untranslated region containing the polyadenylation signal of gene 7 of Agrobacterium T-DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

10	AATCAAGCT TGACGTCAGG TGGCACTTTT CGGGGAAATG TGC GC GGAAC CCCTATTGT	60
	TTATTTTCT AAATACATTC AAATATGTAT CCGCTCATGA GACAATAACC CTGATAAATG	120
	CTTCAATAAT ATTGAAAAAG GAAGAGTATG AGTATTCAAC ATTTCCGTGT CGCCCTTATT	180
15	CCCTTTTTTG CGGCATTTTG CCTTCTCTT TTTGCTCACC CAGAAACGCT GGTGAAAGTA	240
	AAAGATGCTG AAGATCAGTT GGGTGCACGA GTGGGTTACA TCGAACTGGA TCTCAACAGC	300
	GGTAAGATCC TTGAGAGTTT TCGCCCCGAA GAACGTTTTT CAATGATGAG CACTTTTAAA	360
20	GTTCTGCTAT GTGGCGCGGT ATTATCCCGT ATTGACGCCG GGCAAGAGCA ACTCGGTCGC	420
	CGCATACACT ATTCTCAGAA TGACTTGGTT GAGTACTCAC CAGTCACAGA AAAGCATCTT	480
	ACGGATGGCA TGACAGTAAG AGAATTATGC AGTGCTGCCA TAACCATGAG TGATAACACT	540
25	GCGGCCAACT TACTTCTGAC AACGATCGGA GGACCGAAGG AGCTAACC GC TTTTTCAC	600
	AACATGGGGG ATCATGTAAC TCGCCTTGAT CGTTGGGAAC CGGAGCTGAA TGAAGCCATA	660
	CCAAACGACG AGCGTGACAC CACGATGCCT GTAGCAATGG CAACAACGTT GCGCAAAC TA	720
30	TTAACTGGCG AACTACTTAC TCTAGCTTCC CGGCAACAAT TAATAGACTG GATGGAGGCG	780
	GATAAAGTTG CAGGACCACT TCTGCGCTCG GCCCTTCCGG CTGGCTGGTT TATTGCTGAT	840
	AAATCTGGAG CCGGTGAGCG TGGGTCTCGC GGTATCATTG CAGCACTGGG GCCAGATGGT	900
	AAGCCCTCCC GTATCGTAGT TATCTACACG ACGGGGAGTC AGGCAACTAT GGATGAACGA	960
35	AATAGACAGA TCGCTGAGAT AGGTGCCCTCA CTGATTAAGC ATTGGTAACT GTCAGACCAA	1020
	GTTTACTCAT ATATACTTTA GATTGATTTA AAATTCATT TTTAATTTAA AAGGATCTAG	1080
	GTGAAGATCC TTTTGGGCTC GAGTCTCATG ACCAAAATCC CTTAACGTGA GTTTTCGTTT	1140
40	CACTGAGCGT CAGACCCCGT AGAAAAGATC AAAGGATCTT CTTGAGATCC TTTTTTCTG	1200
	CGCGTAATCT GCTGCTTGCA AACAAAAAAA CCACCGCTAC CAGCGGTGGT TTGTTTGCCG	1260
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45	AATACTGTCC TTCTAGTGTA GCCGTAGTTA GGCCACCACT TCAAGAACTC TGTAGCACCG	1380
	CCTACATACC TCGCTCTGCT AATCCTGTTA CCAGTGGCTG CTGCCAGTGG CGATAAGTCG	1440
	TGTCTTACCG GGTGGAGTC AAGACGATAG TTACCGGATA AGGCGCAGCG GTCGGGCTGA	1500
50	ACGGGGGGTT CGTGACACA GCCCAGCTTG GAGCGAACGA CCTACACCGA ACTGAGATAC	1560
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EP 0 757 102 A1

	CCGTAAGCG GCAGGTCGG AACAGGAGAG CGCACGAGGG AGCTTCCAGG GGGAAACGCC	1680
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	TGCTCGTCAG GGGGCGGAG CCTATGGAAA AACGCCAGCA ACGCGGCCTT TTTACGGTTC	1800
	CTGGCCTTTT GCTGGCCTTT TGCTCACATG TTCTTTCCTG CGTTATCCCC TGATTCTGTG	1860
10	GATAACCGTA TTACCGCCTT TGAGTGAGCT GATACCGCTC GCCGCAGCCG AACGACCGAG	1920
	CGCAGCGAGT CAGTGAGCGA GGAAGCGGAA GAGCGCCCAA TACGCAAACC GCCTCTCCCC	1980
	GCGCGTTGGC CTGATCAGAA TTCATATGCA CGTGTTCCTG ATCTAGTAAC ATAGATGACA	2040
	CCGCGCGCGA TAATTTATCC TAGTTTGCGC GCTATATTTT GTTTTCTATC GCGTATTAAA	2100
15	TGTATAATTG CGGGACTCTA ATCATAAAAA CCCATCTCAT AAATAACGTC ATGCATTACA	2160
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	CGGCAACAGG ATTCAATCTT AAGAACTTT ATTGCCAAAT GTTTGAACGA TCTGCTTCGG	2280
20	AGGTTACCTT ATCTGATTTT TGTAAGGTC TGATAATGGT CCGTTGTTTT GTAAATCAGC	2340
	CAGTCGCTTG AGTAAAGAAT CCGGTCTGAA TTTCTGAAGC CTGATGTATA GTTAATATCC	2400
	GCTTCACGCC ATGTTCTGCC GCTTTTGCCC GGGAGTTTGC CTTCCCTGTT TGAGAAGATG	2460
25	TCTCCGCCGA TGCTTTTCCC CGGAGCGACG TCTGCAAGGT TCCCTTTTGA TGCCACCCAG	2520
	CCGAGGGCTT GTGCTTCTGA TTTTGTAATG TAATTATCAG GTAGCTTATG ATATGTCTGA	2580
	AGATAATCCG CAACCCCGTC AAACGTGTTG ATAACCGGTA CCATCGCGAC GGCTTGATGG	2640
30	ATCTCTTGCT GGACACCGGG ATGCTAGGAT GGGTTATCGT GGCCGGCGTG CGTGTGTGGC	2700
	TTTTGTAGGC GCCGGCGACG GCGGGGGCAA TGTGGCAGGT GAGTCACGGT GCAAGCGTGC	2760
	GCAAGTGACT GCAACAACCA AGGACGGTCA TGGCGAAAGC ACCTCACGCG TCCACCGTCT	2820
35	ACAGGATGTA GCAGTAGCAC GGTGAAAGAA GTGTGTCCC GTCCATTAGG TGCATTCTCA	2880
	CCGTGCGCA GAACAGGACC GTTCAACAGT TAGGTTGAGT GTAGGACTTT TACGTGGTTA	2940
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50	GGAAATTATT CAGAATTAGT GCCTTTTATC ATAACTTCTC TCTGAGCCGA TGTGGTTTTG	3480
	GATTTCAATTG TTGGGAGCTA TGCAGTTGCG GATATTCTGC TGTGGAAGAA CAGGAACTTA	3540
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EP 0 757 102 A1

	TCTGCGGGGG TCCTTGCTGG GGCAACATTG ATATGGTTCC TGTTGATGT AGTAGAATAC	3600
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5	TCAAATGCCG CACCACTCTT GGACAGGTAT TAGCTTTATT TCCTGTGGAG ATGGTAGAAA	3720
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	GGTATTCCAG ATAAATATTA AATTTTAATA AAACAATCAC ACAGAAGGAT CTGCGGCCGC	4320
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	CGGTATATAC GATGACTGGG GTTGTAACAA GGCGGCAACA AACGGCGTTC CCGGAGTTGC	4500
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30	TCAGCAACA GACAGGTTGA ACTTCATCCC CAAAGGAGAA GCTCAACTCA AGCCCAAGAG	4620
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	TATGTTACC ACTGATAATG AGAAGGTTAG CCTCTTCAAT TTCAGAAAGA ATGCTGACCC	4860
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40	TCTCCAGGAG ATCAAATACC TTCCCAAGAA GGTTAAAGAT GCAGTCAAAA GATTCAAGAC	4980
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	TCAAAGATAC AGTCTCAGAA GACCAAAGG CTATTGAGAC TTTTCAACAA AGGATAATTT	5340
50	CGGGAAACCT CCTCGGATTC CATTGCCAG CTATCTGTCA CTTTCATCGAA AGGACAGTAG	5400
	AAAAGGAAGG TGGCTCCTAC AAATGCCATC ATTGCGATAA AGGAAAGGCT ATCATTCAAG	5460
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EP 0 757 102 A1

5 ATGCCTCTGC CGACAGTGGT CCCAAAGATG GACCCCCACC CACGAGGAGC ATCGTGGAAA 5520
 AAGAAGACGT TCCAACCACG TCTTCAAAGC AAGTGGATTG ATGTGACATC TCCACTGACG 5580
 TAAGGGATGA CGCACAATCC CACTATCCTT CGCAAGACCC TTCCTCTATA TAAGGAAGTT 5640
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 10 CATGCCGGCG GTCTGCACCA TCGTCAACCA CTACATCGAG ACAAGCACGG TCAACTTCGG 5820
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 15 GAAGGCACGC AACGCCTACG ACTGGACGGC CGAG ACC GTGTACGTCT CCCCCGCCA 6000
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 20 CGAGGCGCTC GGATATGCCC CCCGCGGCAT GCTGCGGGCG GCCGGCTTCA AGCACGGGAA 6180
 CTGCCATGAC GTGGGTTTCT GGCAGCTGGA CTTAGCCTG CCGGTACCGC CCCGTCCGGT 6240
 CCTGCCGTC ACCGAGATCT GAGATCACGC GTTCTAGGAT CCCCCGATGA GCTAAGCTAG 6300
 25 CTATATCATC AATTTATGTA TTACACATAA TATCGCACTC AGTCTTTCAT CTACGGCAAT 6360
 GTACCAGCTG ATATAATCAG TTATTGAAAT ATTTCTGAAT TTAACTTGC ATCAATAAAT 6420
 TTATGTTTTT GCTTGGACTA TAATACCTGA CTTGTTATTT TATCAATAAA TATTTAACT 6480
 30 ATATTTCTTT CAAGATGGGA ATTAACATCT ACAAATTGCC TTTTCTTATC GACCATGTAC 6540
 GTATCGCG 6548

(2) INFORMATION FOR SEQ ID NO: 3:

35 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1601 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear
 40 (ii) MOLECULE TYPE: DNA (genomic)
 (iii) HYPOTHETICAL: NO
 (iv) ANTI-SENSE: NO
 45 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: T72 promoter region
 (ix) FEATURE:
 (A) NAME/KEY: -
 50 (B) LOCATION: complement (1..1601)
 (D) OTHER INFORMATION: /label= PT72
 /note= "promoter region of T72 gene of rice"

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

5	CGCCGTGAGT GTCTTCTGCC GCCGAGGGGC TCTCGCTCGT CGTCGATGCC TGCACGGTGC	60
	GTGCGTGTGT GTCGTGGTGG TGGTGGCGAT ACGCGACGCG AGCTCGATTT ATAGGAGGGG	120
	ATCGAAGGAG GGGAGCGCGC GCGGCGAGGC CCGCGTTGCT CACCTACGCC GCGCGCATGC	180
10	GGCGGACGCG CGGTCGGCGC CCGCGCCGGC CGGGAGGACG AGGGCGCAAG CGTGTGAGCC	240
	ACCGAACGCG CGCGCGCGCC GCGGCGCGAA CTCTCCATCG CGTCGCGGCG AGCCGAGAGC	300
	CGACGAGAGC GTTTCGCGCG CGCGGTTGGG CCGGCGACAA GATGGGCCGT AGCCCTGGGC	360
15	CTCGTGCCAT CTTTTTTTTT CTTTTTTGCC TTTTTTGCC TGGCAATTC TTTTTGTTT	420
	TAGTCTTTTT GTGGTGATAA TGTGTCGTCT TCCGGTGAAC TAATTACTC GTTCATCTTT	480
	TTGTGTCCCT TCGAATATTC GCAGTGGTAG AAGATGACTA CTACTACCAG TAGTTGATCT	540
20	CGAATGGCAA CTTTGTGCA GAACTTATTC CACGGCTATG TCAGCTTCCA CTGTGACTAA	600
	AAAAACTACG GCCATCTTTT GGAATTGTTT TATCTGGAA CTGAACAAA AGGACGATCC	660
	TGATGTACAC ACGGCATAGT TTCCAGCACT GGATGCCAAG TTGCCAACTG TTACCACGAT	720
25	AATGGAACGA CGAGATGAGA TATTATACAA GTCCAATGGA TCAAGATCCT GTGCAGTTGT	780
	TATTGTAAGT GTAACCTAAG CCGTTAATCAT GTACATCACA TTTCCTACTC TATCAATGTC	840
	TTGTGCGGGT TGTTCAAAA AACATGTAC ATCACATGAT CTAGAACGGA AGGCCAGGAT	900
30	ATGAAGTGGT ACTGCAGCAA AACACTGTA GCAGAGATGT ACTATTATGC ATGTACTGTA	960
	GCAGTCATCT AGAGCCGTTG GATCTGAAAA CGAATGGACA TGATTGTGTG CAGTTGCTAT	1020
	TGTGCAGTTA CAATAGCAAC TGCATTTGAT CTTAATCCAA GTCCAATACA TGCAGAACAG	1080
35	TAGCTACGAG CTGGAAAGGA TGCAAATCTG GGTGACACTG ACAGCAACCG TGGAAGAACA	1140
	ACAGCAGCAA AGTCCCAGAG GGATGGCAAT TTGAAGGAAT TTAAATACTC TAATATTACT	1200
	CCACCCGTTA AAAAAACAA CTTGCTACGC ATAATATATG TTCGGATTTA TAGCGAGAAG	1260
40	TTAATTTTTC ATGAGAAGAA GAATATATAT GTAATATGTA CTAGGAGAGT ACTCGCTTCA	1320
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	ATTGTGCAAA TTTTAAATTT TGAAGTAGTT AGATTATCTT TCTAGTAGTT CTGATTGGTT	1440
45	GAAAATGTTT AGATTTTCAT GTGTTAAGAG TTCCGTATCC TAAAAATAGT AATATAATTT	1500
	TAAATCATAT ATATATATAT ATATATATAT ATATATATAT ATATATATAT ATATATATAT	1560
50	TGTTGAACGG TTTGTGCTCT GGTGCTATC CTGTTCTGTG G	1601

55 Claims

1. A process for producing transgenic eucaryote cells which comprises:

contacting a culture of untransformed cells with an inhibitor of poly-(ADP-ribose) polymerase for a period of

time sufficient to reduce the response of the cultured cells to stress and to reduce the metabolism of said cultured cells;

contacting said untransformed cells with foreign DNA comprising at least one gene of interest under conditions in which said foreign DNA is taken up by said untransformed cells and said gene of interest is stably integrated in the nuclear genome of said untransformed cells to produce said transgenic cells; and

optionally recovering said transgenic cells from said culture.

2. The process of claim 1 in which said eucaryotic cells are plant cells.
3. The process of claim 1 or 2 in which said inhibitor is niacinamide, preferably at a concentration of about 150 mg/l to 1000 mg/l, more preferably at a concentration of about 200 mg/l to 500 mg/l, particularly at a concentration of about 250 mg/l.
4. The process or any one of claims 1 to 3 in which said untransformed cells are cultured in a medium containing said inhibitor for a period of time of approximately 2 to 28 days, preferably approximately 3 to 14 days, particularly approximately 4 days prior to the contacting with said foreign DNA.
5. The process of any one of claims 1 to 4 in which said cells contacted with said foreign DNA are further cultured in a medium containing said inhibitor for a period of time of approximately 1 to 14 days, preferably 2 to 4 days after contacting with said foreign DNA.
6. The process of any of claims 1 to 5 in which said gene of interest comprises a promoter that directs expression selectively in certain cells or tissues of an eucaryotic organism.
7. The process of any of claim 2 to 6 in which said gene of interest comprises a promoter that direct expression selectively in stamen cells, particularly anther cells, of a plant.
8. The process of claim 6 or 7 in which said gene of interest encodes a protein that, when produced in a cell of an eucaryotic organism, kills or disables said cell.
9. The process of claim 8 in which said gene of interest encodes a ribonuclease, particularly barnase.
10. The process of any of claims 1 to 9 in which a transgenic organism having said foreign DNA with said at least one gene of interest stably integrated in its genome is obtained from said transformed eucaryotic cell.
11. The process of claim 10 in which said organism is a plant which is obtained by regeneration from a transformed plant cell.
12. The transgenic organism obtained by the process of claim 10 or claim 11.
13. A plant having in the nuclear genome of its cells foreign DNA integrated only in the regions of the nuclear genome that are transcriptionally active in said cells of said plant when said cells are treated with an effective amount of a PARP inhibitor for a period of time sufficient to reduce cell metabolism to a state where gene expression is essentially limited to genes expressed irrespective of the differentiated or physiological condition of the cell.
14. A plant according to claim 13, whereby said integration of the foreign DNA in said transcriptionally active region is verified by measuring the level of expressed mRNA corresponding to this foreign DNA when said cells are incubated in a medium containing PARP.
15. A plant according to claim 13, wherein said transcriptionally active regions of the genome of said plant include regions which are minimally affected by cell differentiation or cell physiological and biochemical changes caused by external factors such as environmental conditions, especially stress conditions.
16. A plant according to any one of the preceeding claims, wherein said plant is a monocotyledonous plant.
17. A plant according to claim 16, wherein said plant is a cereal plant.
18. A plant according to claim 17, wherein said plant is wheat.

19. A plant according to anyone of the preceeding claims, wherein said foreign DNA comprises a DNA sequence expressed selectively in specific tissues of said plant.

5 20. A plant according to claim 19, wherein said foreign DNA comprises a DNA sequence encoding a cytotoxic molecule.

21. A DNA according to claim 20, wherein said foreign DNA comprises a DNA sequence encoding barnase.

10 22. A eucaryote cell having in the nuclear genome of its cells foreign DNA integrated only in the regions of the nuclear genome that are transcriptionally active in said cells of said plant when said cells are treated with an effective amount of a PARP inhibitor for a period of time sufficient to reduce cell metabolism to a state where gene expression is essentially limited to genes expressed irrespective of the differentiated or physiological condition of the cell.

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European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 95 40 1844

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
D,X	NUCLEIC ACIDS RES 18 (20). 1990. 5981-5988., WALDMAN B C ' ILLEGITIMATE AND HOMOLOGOUS RECOMBINATION IN MAMMALIAN CELLS DIFFERENTIAL SENSITIVITY TO AN INHIBITOR OF POLY-ADP-RIBOSYLATION.' * the whole document *	1,22	C12N15/82 C12N15/79 C12N5/10 A01H5/00
D,A	BIOLOGICAL ABSTRACTS, vol. 92, Philadelphia, PA, US; abstract no. 16692, GIROD P-A, ET AL. 'SECONDARY METABOLISM IN CULTURED RED BEET BETA-VULGARIS L. CELLS DIFFERENTIAL REGULATION OF BETAXANTHIN AND BETACYANIN BIOSYNTHESIS.' * abstract *	1-22	
A	& PLANT CELL TISSUE ORGAN CULT 25 (1). 1991. 1-12., --- THE PLANT CELL, vol. 6, no. 3, March 1994 pages 417-426, MLYNAROVA, L., ET AL. 'Reduced position effect in mature transgenic plants conferred by the chicken lysozyme matrix-associated region' * the whole document *	1-22	TECHNICAL FIELDS SEARCHED (Int.Cl.6) C12N A01H
A	THE PLANT CELL, vol. 5, no. 6, June 1993 pages 603-613, ALLEN, G.C., ET AL. 'Scaffold attachment regions increase reporter gene expression in stably transformed plant cells' * the whole document *	1-22	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 22 January 1996	Examiner Maddox, A
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure F : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

EPO FORM 1503 03.92 (P4C01)



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 95 40 1844

DOCUMENTS CONSIDERED TO BE RELEVANT				
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)	
A	PLANT MOLECULAR BIOLOGY, vol. 17, 1991 pages 49-60, PEACH, C., ET AL. 'Transgene expression variability (position effect) of CAT and GUS reporter genes driven by linked divergent T-DNA promoters' * the whole document *	1-22		
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 88, 1991 WASHINGTON US, pages 5212-5216, KERTBUNDIT, S., ET AL. 'In vivo random beta-glucuronidase gene fusions in Arabidopsis thaliana' * page 5215, right column, last paragraph *	1-22		
A	COMPTES RENDUS DE L'ACADEMIE DES SCIENCES SERIE III SCIENCES DE LA VIE 318 (1). 1995. 121-128., DEVIC M., ET AL. 'Assessment of promoter trap as a tool to study zygotic embryogenesis in Arabidopsis thaliana.' * page 124, right column *	13,22		TECHNICAL FIELDS SEARCHED (Int.Cl.6)
A	CHEMICAL ABSTRACTS, vol. 123, no. 21, 20 November 1995 Columbus, Ohio, US; abstract no. 276968, MANDAL, ABUL 'Identification of Arabidopsis thaliana sequences responsive to low temperature and abscisic acid by T-DNA tagging and in-vivo gen fusion' * abstract * & PLANT MOL. BIOL. REP. (1995), VOLUME DATE 1995, 13(3), 243-54, -----	13,22		
The present search report has been drawn up for all claims				
Place of search THE HAGUE		Date of completion of the search 22 January 1996	Examiner Maddox, A	
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